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Inactivation of Naturally Occurring Enteroviruses

Miami Univ., FL

Prepared for

Municipal Environmental Research Lab.
Cincinnati, OH

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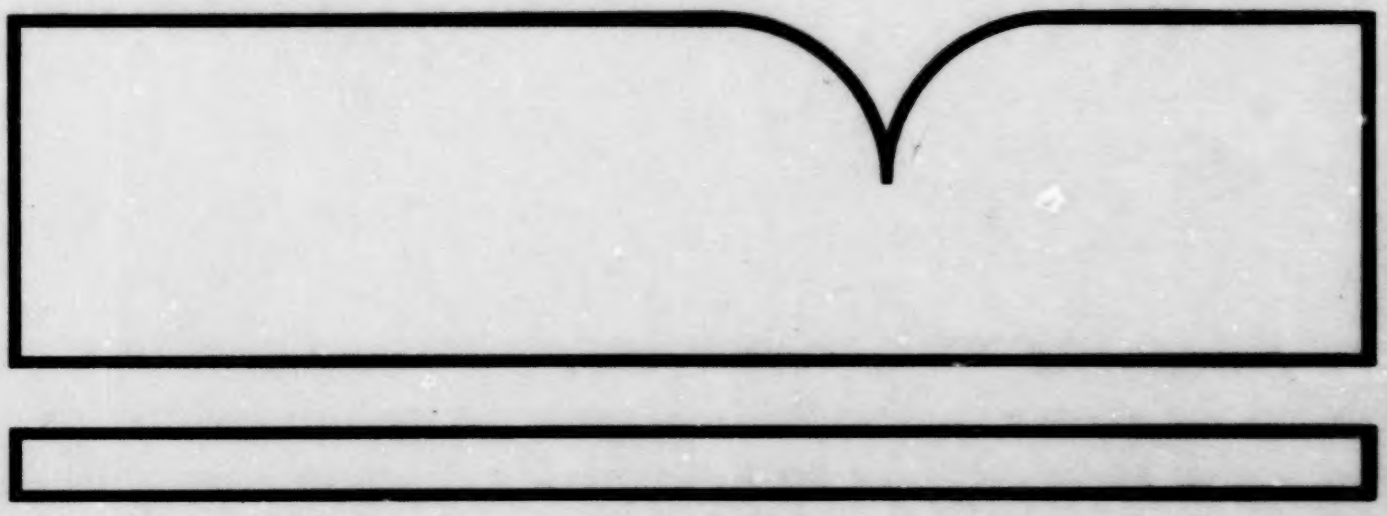

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16. ABSTRACT

The goal of this project was to compare the kinetics of chlorine inactivation of a naturally-shed virus and its tissue culture grown counterpart. Since inactivation studies require purified preparation possessing high infectivity titer and low chlorine demand a major part of this project was devoted to elucidate the best scheme of obtaining material with the necessary criteria.

The scheme found most satisfactory was a combination of Freon 113 extraction, zonal sucrose gradient centrifugation (T1-15 rotor, 25,000 rpm for 3 hours) and pelletization (SW 27 rotor, 27,000 rpm for 3 hours). Using this scheme purified virus preparations derived from naturally shed or tissue culture source were tested for their susceptibility to chlorine inactivation. Preliminary results show that the rate of chlorine inactivation differs with virus derived from fecal material (naturally shed) and that derived from tissue culture. The rate of inactivation indicates that the tissue culture derived virus is more susceptible to chlorine inactivation as compared to naturally shed virus.

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INACTIVATION OF NATURALLY OCCURRING ENTEROVIRUSES

by

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimze the adverse economic, social, health and aesthetic effects of pollution. This publication is one of the products of that research; a most vital communications link between the researcher and the user community.

This research was directed toward comparing the innate chlorine resistance of naturally occurring enteroviruses and cell culture grown progeny of these viruses. A large portion of the effort was devoted to development of methods for separation of naturally occurring viruses from fecal material and this was successful. Because of a series of events that occurred during the final year of the project, including loss of all key personnel and the untimely death of the Principal Investigator, only preliminary inconclusive results on comparaive chlorine resistance were obtained.

Francis T. Mayo, Director
Municipal Environmental
Research Laboratory

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This report is submitted in fulfillment of EPA Grant No. 804780 by the University of Miami School of Medicine under the sponsorship of Microbiological Treatment Branch, Drinking Water Research Division, Municipal Environmental Research Laboratory of the U.S. Environmental Protection Agency. This report covers the period of October 1976 - February 1979.

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IN MEMORIAM

This final report is dedicated to the memory of our beloved colleague, Annie R. Beasley, the principal investigator, whose untimely death occurred September 6, 1978.

This report is a reflection of her long dedication to virological research for the ultimate benefit of mankind.

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SECTION 1

INTRODUCTION

Water pollution by viruses is obviously of great concern to the public and therefore much effort is being devoted by health and environmental agencies to obtain safe water supplies. To achieve this goal various physical procedures and a number of disinfecting agents are used. A very popular disinfecting agent is chlorine. Although much is known about chlorine activity there is a need for more knowledge about the requirements for virus inactivation and yet avoidance of excessive levels of chlorine. The data on virus chlorine inactivation has been generated by two approaches: treatment of wastewater or treatment of tissue culture derived viruses. In the former approach, naturally occurring viruses are usually contained in a milieu of organic and inorganic matter of variable and unknown quality and quantity. The second approach permits assessments of virus inactivation in a "pure" state - but the viruses themselves are not the naturally occurring viruses associated with water or wastewater. A fundamental question remaining unanswered is: are naturally occurring viruses innately of equal, lower or higher susceptibility to chlorine inactivation compared to the same virus types derived from tissue culture? To answer this question the principal investigator, Annie R. Beasley had proposed to purify, identify and quantify virus obtained from fecal filtrates, and these would then be studied for the kinetics of chlorine inactivation. Such information is needed by virologists in general and environmental virologists in particular, as they often accept results obtained with viruses isolated from cell culture. Results in disinfection studies are usually based on viruses obtained from cell culture added to the sludge (spiked) rather than with naturally shed viruses. We have succeeded in preparing from fecal matter purified virus samples which had the necessary high infectivity titer and low chlorine demand needed for chlorine inactivation studies. Unfortunately, the principal investigator, Annie R. Beasley died before completing this project. Wolf Lichter, a member of the same department agreed to complete the project. Because of the very limited time and funds the completion was to be limited to chlorine inactivation of previously purified virus samples and collating the results obtained through the efforts of A. R. Beasley, Principal Investigator; M. M. Sigel, Consultant; and F. Breakenridge, Research Assistant.

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Because of low infectivity titers in the purified virus samples from fecal material the inactivation study by Lichter, et al., was unsuccessful and therefore this report represents only data obtained prior to the untimely death of the original principal investigator, A. R. Beasley.

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SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

We are in need to avail ourselves of the best and safest method for Wastewater disinfection. A commonly found contaminant in wastewater sludge is the enteric group of viruses, therefore studies on the inactivation of these viruses is pursued from many aspects (5, 6, 7). Our results call attention to a very important problem. The problem is one of inferring results obtained with spiked sludge to that of naturally contaminated sludge. Our results show that virus obtained from stools of vaccinated children exhibited more resistance to chlorine inactivation compared to tissue culture derived counterpart. These results are very important especially since most of the data on virus inactivation is not based on naturally shed virus but employs tissue culture derived virus added to sludge.

A great deal of preliminary work towards testing of purified naturally shed virus was accomplished, but the kinetics of chlorine inactivation of the naturally shed virus is preliminary due to the untimely death of the investigator. The results reported here on the purification scheme which resulted in high titered virus and low chlorine demand and the kinetics of chlorine inactivation of these preparations make a follow up study necessary. In fact, before physical or chemical inactivation studies are pursued the susceptibility of the naturally shed virus compared to the tissue culture derived virus must be determined.

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SECTION 3

MATERIALS AND METHODS

VIRUS PURIFICATION ATTEMPTS

The initial purification schemes used normal feces spiked with tissue culture derived poliovirus type 1. The spiked samples were prepared by addition of known amounts of poliovirus ($10^{6.3}$ to $10^{6.5}$ $TCID_{50}$) to 10% homogenates of normal feces.

The initial purification steps included clarification by low speed centrifugation and filtration of the resulting supernatant through sterile stacked AP20, 800 nm and 450 nm Millipore membranes separated by nylon mesh pretreated with 0.3% bovine serum albumin. Virus infectivity was monitored at various steps of the purification procedures.

We have used three purification procedures as outlined in our original proposal:

Procedure 1, after Kapikian et al. (1) as used for human gastroenteritis virus

Centrifuge 3000 rpm for 2 hours.
Filter supernate in an apparatus containing a 1200 nm filter, a dacron mesh and a 450 nm Millipore filter.
Measure infectivity and chlorine demand.
Concentrate and purify by sucrose gradient centrifugation in zonal centrifuge.

Procedure 2, after Bradley (2) as used for hepatitis virus

Clarify by centrifugation at 1000 x g for 30 minutes.
Centrifuge supernates at 12,000 x g for 30 minutes.
The supernate was further purified by (a) passing through a 450nm Millipore membrane (b) adsorption/elution from bentonite or (c) adsorption/elution with PE60 or membranes.
The product with highest infectivity and lowest chlorine demand was subjected to further purification and concentration by molecular sieve filtration through a Millipore Pellicon Cassette system fitted with a PTGC membrane. Alternatively, concentration can be effected by filtration through an Amicon

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ultrafiltration device fitted with a PM-30 membrane.
Purify by sucrose gradient centrifugation or by aqueous polymer 2-phase separation.

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Procedure 3, after Petric et al. (3)

Extract fecal matter in phosphate buffered saline (PBS).
Homogenize for 2 minutes at full speed in a Virtis homogenizer.
Clarify by centrifugation at 3000 x g for 10 minutes.
Ultracentrifuge at 110,000 x g for 2 hours.
Resuspend pellet in PBS to 1:40 of the original volume and extract with 0.25 volume of Freon 113.
Centrifuge aqueous phase of extract at 110,000 x g for 2 hours.
Resuspend pellitized virus in a small volume of PBS.

The Adopted Method of Purification

The method shown below was an adaptation of the above procedures and was chosen for extracting, purification and concentration based on results with normal feces spiked with poliovirus type 1. This procedure when applied to pooled post-vaccine fecal samples and tissue culture derived virus yielded preparations that (i) possessed high infectivity and (ii) had relatively low chlorine demand.

1. Prepare a 20% (wt/vol) homogenate of stool in 0.05 M PBS, pH 7.2.
2. To each 100 ml add 25 ml Freon 113. Shake 1 hour.
3. Centrifuge 1000 x g for 30 minutes. Harvest aqueous phase.
4. Discard Freon; replace with 25 ml Freon. Add additional PBS in an amount equal to the initial volume of homogenate.
5. Shake and centrifuge as above.
6. Harvest aqueous phase and pool with initial harvest.
7. Filter extract through sterile stacked AP20, 800 nm and 450 nm Millipore membranes separated by nylon mesh pretreated with 0.3% bovine serum albumin.
8. If necessary, reduce volume by hydroextraction, using PEG 20,000.
9. Layer 100 ml on a preformed sucrose gradient (10-30% in 0.05 M PBS) in a T1-15 zonal rotor (Beckman). Centrifuge at 25,000 rpm for 3 hours. Unload from center, collecting 50 ml fractions.
10. Pool fractions with highest virus titers. Dialyze overnight against 0.05 m PBS pH 7.2, 25 x volume of pool.
11. Pelletize by centrifugation (Beckmsn SW-27 rotor) at 27,000 rpm for 3 hours.
12. Resuspend pellets in phosphate buffer (PB) pH 6.0, and freeze at -20°C.

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CHLORINE AND INACTIVATION PROCEDURE

A stock preparation of sodium hypochlorite was prepared from household bleach and the residual free chlorine determined by amperometric titration. This stock preparation contained 9400 mg free chlorine per liter and was used to determine the chlorine demand of all our preparations.

For determination of chlorine demand, virus preparations were diluted 1:100 in chlorine demand-free water. To 10 ml portions, sodium hypochlorite was added with a Hamilton syringe to give a final concentration of 1 mg chlorine per liter. After a 20-30 minute contact period at room temperature, residual free chlorine was measured by the DPD colormetric method given below:

Reagents

a. Phosphate buffer solution (PB): Dissolve 24 g anhydrous disodium hydrogen phosphate, NA_2HPO_4, in distilled water. Combine with 100 ml distilled water in which 800 mg disodium ethylenediamine tetraacetate dihydrate, has been dissolved. Dilute to 1 l with distilled water and add 20 mg $HgCl_2$ to prevent interference in the free available chlorine test caused by any trace amounts of iodide in the reagents.

b. N,N-Diethyl-p-phenylenediamine (DPD indicator solution): Dissolve 1 g DPD Oxalate or 1.5 g p-amino-N:N-diethylaniline sulfate, in chlorine-free distilled water containing 8 ml 1+3 ml H_2SO_4 and 200 mg disodium ethylenediamine tetraacetate dihydrate, also called (ethylenedinitrilo) tetraacetic acid sodium salt. Make up to 1 l, store in a brown glass-stoppered bottle, and discard when discolored. (The buffer and indicator sulfate are commercially available. The Oxalate is Eastman chemical No. 7102, or equivalent).

c. Free chlorine: Place 0.5 ml each of buffer reagent and DPD indicator reagent in a test tube or photometer cell. Add 10 ml sample and mix. Read the color immediately in colorimeter at 515 nm.

We set up chlorine inactivation experiments aiming for an initial concentration of chlorine of 0.5 mg/liter. The chlorine inactivation experiments were set up as follows:

(i) A stock of chlorine demand free 0.05 M PB pH 6.0 was prepared and placed in an ice bath.
(ii) To half of the PB chlorine was added to a concentration of approximately 0.5 mg/liter and kept in an ice bath. (= PB + Cl).
(iii) Free chlorine was determined by the DPD method.

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(iv) 2 x 100 ml aliquots of PB + Cl and 1 x 100 ml aliquots of PB in 200 ml beakers in ice bath were removed and placed on magnetic stirrers - rates of spinning were adjusted to match each other.

(v) 1.0 ml of "purified" virus was added to one beaker containing 100 ml of PB + Cl and 1.0 ml of "purified" virus was added to the beaker with 100 ml of PB. 1.0 ml of PB was added to the second beaker containing 100 ml of PB + Cl. Immediately after the addition of these 1.0 ml samples, 10 ml aliquots of each was assayed for residual free chlorine using the DPD method ('0' time).

(vi) From the beaker containing 1.0 ml of purified virus and 100 ml of PB + Cl 5.0 ml samples were collected every minute for 10 minutes and placed in tubes containing 0.5 ml of 0.06% sodium thiosulfate (STF) solution to neutralize residual chlorine.

(vii) From the beaker containing 1.0 ml "purified" virus in 100 ml of PB 5.0 ml samples were removed and placed in tubes containing 0.5 ml STF solution 0.06% at the beginning and end of experiment. These samples were used as virus controls.

(viii) At the end of 10 minutes samples remaining in beakers were checked for residual chlorine and pH. All 5.0 ml samples in 0.06% STF solution were frozen at -20°C for future viral assay. All samples for each experiment were assayed for viral activity on the same day and with the same batch of tissue culture cells.

VIRUS INFECTIVITY ASSAYS

Virus infectivity was determined by microscopic evaluation of observed cytopathogenic effect (CPE) in tissue cultures of Buffalo Green Monkey (BGM) kidney cells. Tube cultures of BGM cells were initiated in Medium 199 with 10% heat inactivated fetal calf serum (FCS), (growth medium) at 80,000 to 100,000 cells per tube (1 to 1.2 ml). After 2-3 days the cells were confluent. The spent culture media were decanted and the monolayer tubes were refed with M 199 with 2% heat inactivated FCS (maintenance medium). Then 0.1 ml of virus dilution was inoculated into replicate tubes (5 or more) for each dilution with appropriate control tubes. These cultures were then incubated at 37°C and observed microscopically and scored daily for CPE. After 7 days incubation and scoring the experiment was terminated. The titers were calculated using the Reed-Muench (3) formula for 50% end points.

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RESULTS AND DISCUSSION

To obtain purified virus preparations for the chlorine inactivation study 3 purification procedures were used. The initial purification studies used 10% homogenates of normal feces spiked with tissue culture derived poliovirus type 1. In all procedures the initial steps included clarification by low speed centrifugation and filtration of the resulting supernatant through stacked AP20, 800 nm and 450 nm membranes. Portions of filtrates were frozen for titration and the purification procedures were monitored at various steps by freezing samples for virus infectivity assays when appropriate.

The results obtained by purification Scheme 1 (centrifugation and Freon extraction) are shown in Table 1. The titer of recovered virus was satisfactory but the chlorine demand was high.

Scheme 2 used a 10-30% sucrose gradient prepared in PBS. The results of the zonal sucrose gradient centrifuging action of the spiked fecal homogenate are shown in Table 2. The maximum virus concentration was found in fractions 5 through 10. However, it is to be noted that most fractions demonstrated infectious virus.

Scheme 3 PEG 6000 was added to the crude filtrate (see Materials and Methods) and the results are shown in Table 3. The maximum virus titers were found in fractions 8 and 10. Infectious virus was demonstrable in all fractions except the last one. This last fraction was used to resuspend some pelletized material, and the preparation was toxic for the assay cultures at low dilutions.

To determine the chlorine demand of the various virus preparations sodium hypochlorite was used. The concentration of free chlorine and the uptake by the virus was determined by amperometric titration. Continued attempts were made toward obtaining high titered preparations of naturally shed poliovirus(es) with low chlorine demand. For this purpose stools of children recently immunized with oral poliovirus vaccine were used as source material. Homogenates of these feces were prepared in phosphate buffered saline (PBS) and clarified by low speed centrifugation. The resulting supernates were

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TABLE 1. CONCENTRATION OF POLIOVIRUS FROM SPIKED FECAL HOMOGENATES BY ULTRACENTRIFUGATION AND FREON 113 EXTRACTION

Lot No.	Crude Homogenate Volume	Crude Homogenate Titer	Final Preparation Volume	Final Preparation Titer	Chlorine Demand
I	77 ml	$10^{6.3}$ ID_{50}/ml	2 ml	$10^{7.5}$ ID_{50}/ml	0.54 mg/l
II	40 ml	$10^{6.5}$ ID_{50}/ml	2 ml	$10^{7.75}$ ID_{50}/ml	0.29 mg/l

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TABLE 2. CONCENTRATION OF POLIOVIRUS FROM A SPIKED FECAL HOMOGENATE BY ZONAL SUCROSE GRADIENT CENTRIFUGATION

Fraction Number	Chlorine Demand mg/L	Infectivity[a] at: 10-2	10-4	10-6	Fraction Number	Chlorine Demand mg/L	Infectivity[a] at: 10-2	10-4	10-6
1	ND	-	-	-	17	0.01	-	-	-
2	ND	±	-	-	18	0.01	±	-	-
3	ND	+	-	-	19	0.01	-	-	-
4	0.95	+	-	-	20	0	-	-	-
5	0	+	+	-	21	0	-	-	-
6	0.18	+	+	-	22	0	-	-	-
7	0.13	+	+	-	23	0	±	-	-
8	0.22	+	+	-	24	0.01	-	-	-
9	0.16	+	+	±	25	ND	-	-	-
10	9	+	+	-	26	0.13	-	-	-
11	0.01	+	±	-	27	0.23	±	-	-
12	0	+	-	-	28	0.15	-	-	-
13	0.03	±	-	-	29	0.16	-	-	-
14	0.03	±	-	-	30	0.16	-	-	-
15	0.01	±	-	-	31	ND	-	-	-
16	0	±	-	-	32	0.16	-	-	-

a. Cytopathic effect of 0.1 ml inoculum based on 5 or more cultures per dose:
+ = cytopathic effect in all cultures.
± = cytopathic effect only in some cultures.
- = no cytopathic effect observed.

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TABLE 3. CONCENTRATION OF POLIOVIRUS FROM SPIKED FECAL HOMOGENECITY BY POLYETHYLENE GLYCOL 6000 PRECIPITATION AND SUCROSE GRADIENT CENTRIFUGATION

Fraction Number	Chlorine Demand mg/L	Infectivity* at Dilution: 10^{-1}	10^{-3}	10^{-5}	10^{-6}	10^{-7}
1	0.68	+	±	-	-	-
2	0.65	+	-	-	-	-
3	0.32	+	±	-	-	-
4	0.10	+	+	±	-	-
5	0.10	+	+	-	-	-
6	0.02	+	+	-	-	-
7	0.0	+	+	-	-	-
8	0.02	+	+	+	±	-
9	0.17	+	+	+	-	-
10	0.0	+	+	+	±	-
11	0.07	+	+	±	-	-
12	0.02	+	+	-	-	-
13	0.02	+	+	±	-	-
14	0.07	+	+	-	-	-
15	0.08	+	+	±	-	-
16	0.57	Toxic	Toxic	Toxic	-	-

* See legend in TABLE 2.

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filtered through stacked AP20, 800 nm and 450 nm Millipore membranes, followed by zonal sucrose gradient centrifugation as previously described. Fifty ml fractions were collected from the center and refractive indices determined to assure that a gradient had been established. Each fraction was tested for chlorine demand as previously described and selected dilutions of each were assayed in BGM monolayer cultures for infectivity.

Results from one such zonal density gradient centrifugation are presented in Table 4. Maximum titers ($10^{5.0}$ - $10^{5.5}$ ID_{50}/ml) were in fractions 4-7. These, however, had undesirable chlorine demands and efforts were made to further purify the virus. The 4 fractions were pooled, diluted 1·2 with PBS, and the virus pelletized by centrifugation at 100,000 g for 4 hours. The sedimented virus was resuspended in 10 ml PBS then twice extracted with Freon 113. At each step, the material was monitored for chlorine demand and infectivity. Results of these sequential treatments are summarized in Table 5.

A second 10% stool homogenate was subjected to zonal density gradient centrifugation. Chlorine demand and virus infectivity of each fraction were measured. Results are shown in Table 6. Again, maximum titers of $10^{5.5}$ ID_{50}/ml were obtained, but the chlorine demands were less than those from the experiment cited above.

In another approach to obtain purified preparations the procedure outlined below was used:

1. 60 ml of a 20% stool homogenate was extracted with Freon 113.
2. The mixture was centrifuged at 1,000 g for 30 minutes and the aqueous supernatant was aspirated.
3. The Freon phase and sediment were mixed with 60 ml PBS, centrifuged and the aqueous phase aspirated and mixed with the initial supernatant.
4. The resulting preparation was filtered and 100 ml subjected to zonal density gradient centrifugation.

The results of infectivity assays and chlorine demand determinations are shown in Table 7. This procedure yielded the best results and became a part of our adopted method of purification.

Using this purification method we prepared two samples that had a chlorine demand of 0.12 mg/liter and titered $10^{5.0}$ and $10^{5.75}$ ID_{50}/ml. Along with these samples, polio vaccine used in a volunteer project, was passed once in BGM tissue culture and the viral harvest was purified in a similar manner, except for step 1, where the infected cells were frozen and thawed 3 times and then treated with Freon 113. This "purified" preparation had a chlorine demand of 0.10 mg chlorine per liter and a titer of $10^{6.67}$ ID_{50}/ml. All "purified" preparations were

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TABLE 4. RECOVERY OF NATURALLY SHED VIRUS FROM A STOOL SUSPENSION BY ZONAL SUCROSE GRADIENT CENTRIFUGATION

Fraction Number	Chlorine Demand mg/L	Infectivity* at Dilution: 10^{-2}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
3	ND	ND	ND	±	±	-
4	0.45	+	+	+	+	-
5	0.36	+	+	+	+	-
6	0.36	+	+	+	+	-
7	0.19	+	+	+	±	-
8	0.15	+	+	+	±	-
9	0.15	+	+	±	-	-
10	0.18	+	±	±	-	-
11	0.15	±	-	-	-	-
12	0.23	±	±	-	-	-
13	0.18	±	-	-	-	-
14	0.22	±	-	-	-	-
15	0.12	-	-	-	-	-
16	0.08	-	-	-	-	-

* See legend in TABLE 2.
ND = not done.

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TABLE 5. FURTHER CONCENTRATION AND PURIFICATION OF VIRUS FROM POOLED GRADIENT FRACTIONS BY ULTRACENTRIFUGATION AND EXTRACTION WITH FREON 113

Material tested	Volume (ml)	Titer (ID_{50}/ml)*	Chlorine Demand (mg/L)
Pooled gradient fractions (4-7, TABLE 1)	180	$10^{5.5}$	0.45 - 0.19
Suspension of pellitized virus	10	$10^{7.25}$	0.32
1st extraction with Freon	8	$10^{6.6}$	0.30
2nd extraction with Freon	6	$10^{6.6}$	0.15

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TABLE 6. RECOVERY OF NATURALLY SHED POLIOVIRUS BY ZONAL DENSITY GRADIENT CENTRIFUGATION OF A STOOL EXTRACT

Fraction Number	Chlorine Demand mg/L	Infectivity* at Dilution: 10^{-2}	10^{-3}	10^{-4}
3	0.74	±	-	-
4	0.68	+	-	-
5	0.58	+	-	-
6	0.13	+	±	-
7	0.02	+	±	-
8	0.04	+	+	-
9	0	+	+	-
10	0.02	+	+	-
11	0.04	+	+	-
12	0	+	-	-
13	0.005	±	-	-
14	0	±	-	-
15	0	-	-	-

* See legend on TABLE 2.

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TABLE 7. CONCENTRATION AND PURIFICATION OF NATURALLY SHED VIRUS FROM STOOL HOMOGENATES BY FREON 113 EXTRACTION FOLLOWED BY ZONAL SUCROSE GRADIENT CENTRIFUGATION

I.	Initial 20% suspension (60 ml)	$10^{5.75}$ ID_{50}/ml
II.	Supernate from 1st Freon extraction (42 ml)	$10^{6.0}$ ID_{50}/ml
III.	Supernate from 2nd extraction (62 ml)	$10^{5.0}$ ID_{50}/ml
IV.	Pooled supernates (100 ml used for gradient)	$10^{5.4}$ ID_{50}/ml

V. Sucrose density gradient fractions

Fraction Number	Chlorine Demand mg/L	Log_{10} ID_{50}/ml
12	0	1.5
13	0.03	1.5
14	0	3.5
15	0	3.5
16	0	5.5
17	0.08	4.2
18	0	3.5
19	0	3.0
20	0	1.5
21	0.03	1.5

Highest chlorine demands found in initial fractions.

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frozen at -20°C.

With these "purified" preparations, we proceeded to set up chlorine inactivation experiments aiming for an initial concentration of chlorine of 0.5 mg/liter. Free chlorine was measured using the DPD method previously described. Virus infectivity was measured by inoculating 0.1 ml of each dilution into tube cultures of BGM tissue culture cells. Titers were calculated using the Reed-Muench formula for 50% end points.

The results of the first and second experiment are shown in Table 8. These experiments were done on the same day using the same reagents but different virus preparations. The attempt here was to compare chlorine inactivation of naturally shed virus and tissue culture derived virus. The naturally shed virus here was typed as poliovirus type 1 by neutralization test. As can be seen in Table 8 there appears to be a marked difference in the inactivation rate within the first five minutes between the viral preparations. However, after eight minutes both preparations show no viral activity. We see a loss of free chlorine from 0.46 mg/liter initially to 0.28 mg/liter at the time of the addition of virus samples and to 0.14 and 0.16 mg/liter at the end of the experiments. There was no significant change in pH in the experimental samples. Both experiments were assayed for viral activity on the same day using the same batch of tissue culture cells. The results of these experiments indicated that there is a difference in the chlorine inactivation of naturally shed poliovirus type 1 and tissue culture derived poliovirus type 1.

The results of another inactivation experiment are shown in Table 9. Unfortunately, the viral assay for this experiment was terminated on day 4 due to bacterial contamination of all the tubes in the assay. We normally compute our $TCID_{50}$ titers after 7 days of observation, consequently the results here are inconclusive.

An attempt was made to obtain large amounts of monovalent virus from stool specimens by administration to volunteers type 1 component of Sabin vaccine. The vaccine (Pfizer lot #266, $TCID_{50}$ $1 \times 10^{6.7}$) was suspended in sterile Hank's Balanced Salt Solution (HBSS) and administered orally. Stool samples were collected on days 3 through 14. Ten percent fecal suspensions in HBSS of the samples obtained were treated with chloroform and assayed for virus activity in BGM tissue culture tubes. As shown in Table 10 no virus activity was demonstrated. This was disappointing as we had hoped that the large yield of fecal material obtained from adults would have given us adequate amounts of monovalent virus to work with.

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TABLE 8. COMPARISON OF THE INACTIVATION OF "PURIFIED" NATURALLY SHED (NS) POLIOVIRUS TYPE 1[a] FROM STOOL SUSPENSION AND "PURIFIED" TISSUE CULTURE (TC) DERIVED POLIOVIRUS TYPE 1 BY 0.46 mg/L[c] CHLORINE OVER A TEN MINUTE PERIOD

Time (minutes)	Log ID_{50}/ml[d] NS	Log ID_{50}/ml[d] TC[b]	% Survival NS	% Survival TC	Cl-mg/L NS	Cl-mg/L TC	pH NS	pH TC
0	3.0	3.5	100.0	100.0	0.28	0.28	6.00	6.00
1	2.5	2.17	31.62	4.68	ND	ND	ND	ND
2	2.33	1.82	21.38	2.09	ND	ND	ND	ND
3	1.5	1.24	3.16	0.55	ND	ND	ND	ND
4	1.5	0.37	3.16	0.07	ND	ND	ND	ND
5	1.17	0.5	1.48	0.10	ND	ND	ND	ND
6	0.62	-1.62	0.417	0.13	ND	ND	ND	ND
7	0.62	0	0.417	0	ND	ND	ND	ND
8	0	0	0	0	ND	ND	ND	ND
9	0	0	0	0	ND	ND	ND	ND
10	0	0	0	0	0.14	0.16	6.06	6.04

a Concentration of virus used in test was completely neutralized by polio type 1 antiserum.
b Monovalent polio type 1 oral vaccine-Pfizer, passaged in tissue culture (BGM cells).
c Concentration of chlorine prior to introduction of virus.
d Virus titers based on 7 day observation of cultures.

TABLE 9. COMPARISON OF THE INACTIVATION OF "PURIFIED NATURALLY SHED (NS) POLIOVIRUS TYPE 1[a] FROM STOOL SUSPENSION AND "PURIFIED" TISSUE CULTURE (TC) DERIVED POLIOVIRUS TYPE 1[b] BY 0.48 mg/L[c] CHLORINE OVER A TEN MINUTE PERIOD

Time (minutes)	Log ID_{50}/ml[d] NS	Log ID_{50}/ml[d] TC[b]	% Survival NS	% Survival TC	Cl-mg/L NS	Cl-mg/L TC	pH NS	pH TC
0	2.0	2.0	-	-	0.28	0.38	6.00	6.00
1	0.62	0.62	4.17	4.17	ND	ND	ND	ND
2	0	0.62	0	4.17	ND	ND	ND	ND
3	0	0	0	0	ND	ND	ND	ND
4	0	0	0	0	ND	ND	ND	ND
5	0	0	0	0	ND	ND	ND	ND
6	0	0	0	0	ND	ND	ND	ND
7	0	0	0	0	ND	ND	ND	ND
8	0	0	0	0	ND	ND	ND	ND
9	0	0	0	0	ND	ND	ND	ND
10	0	0	0	0	0.16	0.32	6.05	6.05

a Concentration of virus used in test was completely neutralized by polio type 1 antiserum.
b. Monovalent polio type 1 oral vaccine-Pfizer, passaged in tissue culture (BGM cells).
c. Concentration of chlorine prior to introduction of virus.
d. Observation period limited to 4 days due to fungal contamination of cultures.

TABLE 10. SUMMARY OF ATTEMPT TO RECOVER VIRUS FROM STOOLS OF ADULT MALE VOLUNTEERS IMMUNIZED WITH ORAL POLIO TYPE 1 VACCINE - PFIZER

Volunteers	Day 3 titers[a]	Day 4 titers	Day 5 titers	Day 6 titers	Day 7 titers	Day 12 titers	Day 14 titers
W.L.	10^2	-	10^2	10^2	10^1	-	10^1
L.W.	10^2	10^2	10^2	10^1	-	10^1	-
K.F.	-	10^2	-	10^2	-	-	-
L.R.	10^2	10^2	10^2	10^1	10^1	-	-

a Titers ID_{50}/0.1 ml of 10% stool suspensions

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